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Ototoxicity of Cisplatin, Pyriplatin, and Phenathriplatin in the Auditory Hybridoma Cell Line, HEI-OC1

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OTOTOXICITY OF CISPLATIN, PYRIPLATIN, AND PHENANTHRIPLATIN IN THE AUDITORY HYBRIDOMA CELL LINE, HEI-OC1

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By

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ABSTRACT

Cisplatin is an anti-cancer drug which is effective against several cancers, but also causes harmful side-effects, including ototoxicity and hearing loss. While cisplatin is a bifunctional compound that forms coordinate covalent bonds with both strands of DNA, recently investigated monofunctional platinum(II) compounds bind to only one DNA strand, and may activate different cell-death mechanisms. As several monofunctional platinum(II) compounds have anti-cancer properties, but could target different cell-death pathways, they could potentially have different and reduced side-effects. In this study, the HEI-OC1 auditory hybridoma cell line was used to investigate the ototoxicity of cisplatin and two monofunctional platinum(II) compounds, phenanthriplatin and pyriplatin. First, a colorimetric spectrophotometer assay was used to measure HEI-OC1 cell viability after treatment with cisplatin, phenanthriplatin, or pyriplatin. Next, a fluorescent flow-cytometric assay was used to measure ROS levels. Finally, a luminescent spectrophotometric assay was used to measure caspase-3/7 levels. My results show that at 24- and 48-hours post-treatment, cisplatin, phenanthriplatin, and pyriplatin had similar effects on cell viability. At 24-hours, ROS levels significantly increased for each compound, but at 48-hours, ROS levels were comparable to the control. At 24-hours, caspase-3/7 activity was significantly decreased for each treatment. At 48-hours, caspase-3/7 activity was elevated only for the cisplatin treatment and was not different than the control for both monofunctional compounds.

I dedicate this thesis to my parents, Jeff and JoLynn Johnston, who have always been my greatest support system. I would not be where I am today without them.

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Monroe JD, Johnston AM, Smith ME. 2020. The monofunctional platinum(II) compounds, phenanthriplatin and pyriplatin, modulate apoptosis signaling pathways in HEI-OC1 auditory hybridoma cells. *Neurotoxicology*.

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INTRODUCTION

According to the International Agency for Research on Cancer, there were over 18 million new cancer diagnoses in 2018 worldwide, and over 9.5 million cancer-related deaths (World Health Organization, 2019). And while cancer treatments are continually improving, devastating side-effects are still common. Cisplatin is an antineoplastic drug used to treat many types of cancers, including testicular, bladder, head, neck, lung, ovarian, and cervical cancers, but often causes dose-dependent nephrotoxicity, neurotoxicity, and ototoxicity (Theneshkumar & Hatzopoulos, 2007; Ciarimboli, 2012; Leisching et al., 2015). Ototoxic side-effects include hearing loss, ear pain and tinnitus (Ciarimboli, 2012), caused by sensory hair cell loss in the organ of Corti and other areas of the inner ear (Bhavsar et al., 2017). Therefore, the discovery of novel antineoplastic compounds that are cytotoxic to cancer cells, yet avoid side-effects such as hearing loss, could lead to valuable clinical applications.

The mechanism of action of cisplatin is well characterized. Initially, cisplatin uptake occurs through the copper transporter 1 (Ctr1) located in cell membranes (Ishida et al., 2002). As the cytoplasmic concentration of chloride is usually lower than the extracellular concentrations, water replaces the two chlorides on cisplatin (aquation), creating an electrophile (Karasawa & Steyger, 2015). The aquated form of cisplatin can then enter the nucleus and form a coordinate covalent bond with DNA, creating cisplatin/DNA adducts at the N7 positions of predominantly guanine residues with less frequent binding to adenine residues (Karasawa & Steyger, 2015; Marques et al., 2015).

These adducts typically cause the formation of bifunctional 1,2-intrastrand crosslinks, which cause DNA distortion, and become binding sites for proteins containing high-mobility group (HMG) domains, which inhibit DNA excision repair and lead to the activation of apoptotic pathways (Huang et al., 1994; Cepeda et al., 2007; Guggenheim et al., 2009).

Cisplatin can activate several pathways that cause cell death, including the extrinsic and intrinsic pathways. In general, these processes involve caspases, a group of cysteine proteases present in cells as zymogens until activated (Adams, 2003). The extrinsic pathway is activated when tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo2L) interacts with death receptors four and five, causing the recruitment of the zymogen, procaspase-8, and other proteins, which then form a complex with active caspase-8 (Adams, 2003). Caspase-8 then activates procaspases-3 and -7, converting them into their active forms, caspases-3 and -7, which are "executioner" caspases that cleave various proteins involved with structure and repair, resulting in cell death (Slee et al., 2001; Adams, 2003; Florea & Büsselberg, 2011). Reactive oxygen species (ROS) production can sometimes activate the extrinsic pathway by stimulating pro-death proteins and suppressing NF-κB, a protein involved in a survival pathway (Han et al., 2009).

The intrinsic cell death pathway, which can cause proapoptotic proteins to translocate from the cytoplasm into the mitochondria, can increase outer mitochondrial membrane permeability (Sheth et al., 2017). Disruption of mitochondrial membrane integrity is followed by increased production of ROS as well as the release of Cytochrome c from the mitochondria and then activation of caspase-9, an initiator

caspase, which subsequently activates caspase-3, causing apoptosis (Hengartner, 2000; Sheth et al., 2017).

Cisplatin treatment can also increase reactive oxygen species (ROS) production in the cochlea and cause ototoxicity (Sheth, 2017). When cisplatin binds to mitochondrial DNA, it can inhibit transcription, prevent the synthesis of mitochondrial proteins, and impair the function of the electron transport chain leading to increased ROS (Marullo et al., 2013). Higher levels of ROS can activate the extrinsic and intrinsic apoptosis pathways, and promote ototoxicity (Karasawa & Steyger, 2015). Increased ROS can also modulate cell death mechanisms that act independently of the extrinsic and intrinsic pathways (Karasawa & Steyger, 2015; Redza-Dutordoir & Averill-Bates, 2016). ROS generation can cause the release of the apoptosis-inducing factor (AIF) protein from the mitochondria, which then travels to the nucleus where it can cause DNA fragmentation and chromosome condensation, and caspase-independent apoptosis (Candé, 2002; Redza-Dutordoir & Averill-Bates, 2016; Bano & Prehn, 2018). Increased ROS production from cisplatin treatment can also cause cell death through caspase-12 signaling (Karasawa & Steyger, 2015; Redza-Dutordoir & Averill-Bates, 2016; Zong, 2017). Caspase-12 is stored in the endoplasmic reticulum (ER) and is activated in response to ER stress (Nakagawa, 2000). Once activated, caspase-12 can then activate caspase-9, which activates caspase-3 (Szegezdi, 2003). Modulation of cell death mechanisms in auditory hair cells could lead to hearing and/or balance loss; as human hair cells do not regenerate, this loss is permanent (Cheng et al., 2005).

Monofunctional platinum(II) compounds have different cell uptake and DNA binding properties than cisplatin. Unlike cisplatin, which utilizes the copper transporter

for cell uptake, the monofunctional compound phenanthriplatin primarily enters cells through organic cation transporters (Ishida et al., 2002; Hucke et al., 2018). Although monofunctional platinum(II) compounds have only one chloride leaving ligand, this chloride, like those of cisplatin, becomes aquated after cellular entry, which enables formation of a covalent bond with nucleophilic DNA (Park et al., 2012; Zhou et al., 2018; Guo et al., 2019). As with cisplatin, phenanthriplatin predominately binds to purines, but it has been found to have a greater binding affinity for adenines than cisplatin (Park et al., 2012; Riddell et al., 2016; Zhou et al., 2018). Monofunctional platinum(II) compound adducts can prevent transcription by hindering the progress of RNA polymerase II, and cause apoptosis (Kellinger et al., 2013; Guo et al., 2019). However, monofunctional platinum(II) compounds do not cause DNA distortion as does cisplatin (Guo et al., 2019). As monofunctional platinum(II) compounds do not activate some of the cell death pathways utilized by cisplatin, treatment with these compounds may not cause serious side-effects. Recently, phenanthriplatin and pyriplatin, two monofunctional platinum(II) compounds, were shown to have little or no significant effect on hair cell density in zebrafish auditory endorgans (Monroe et al., 2018). In this study, phenanthriplatin, like cisplatin, caused significant auditory threshold shifts for most of the tested frequencies (Monroe et al., 2018). These results suggest a different mechanism of action of these monofunctional platinum(II) compounds, and potentially reduced ototoxicity, as compared to cisplatin (Monroe et al., 2018).

In these experiments, I worked with the monofunctional platinum(II) compounds pyriplatin and phenanthriplatin. Pyriplatin, or cis-diammine(pyridine)chloroplatinum(II), has been shown to be less potent than cisplatin (Lovejoy et al., 2011), leading to research

to find structurally similar compounds with greater anti-cancer efficacy. Based on the transcription inhibition mechanism of pyriplatin (Wang et al., 2010), phenanthriplatin (cis-[$Pt(NH_3)_2$(phenanthridine)Cl]NO_3) was synthesized, and has been shown to have greater effect than cisplatin against some cancer cell lines (Park et al., 2012; McDevitt et al., 2019).

This study used the mouse auditory hybridoma cell line, House Ear Institute-Organ of Corti 1 (HEI-OC1) to determine whether cisplatin and the two monofunctional platinum compounds, pyriplatin and phenanthriplatin, had similar effects on cell viability, ROS production, and caspase-3/7 activity. This cell line expresses both the molecular and structural characteristics of mammalian auditory cells and has served as a model to study cochlear hair cell ototoxicity mechanisms, including those modulated by cisplatin, and to investigate new drugs for their ototoxic or otoprotective qualities (Du et al., 2013; Kalinec et al., 2016; Li et al., 2018).

In Part I, the MTT assay was used to determine the effect of these compounds on HEI-OC1 cell viability at 24- and 48-hour time points and to derive IC_{50} dosage values for each compound for the subsequent experiments. In Part II, flow cytometry and the fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), was used to measure the effect of these compounds on reactive oxygen species (ROS) production at 24- and 48-hour time points. Finally, in Part III, a luminescent spectrophotometric assay was used to measure how each drug affected caspase-3/7 activity at the 24- and 48-hour time points.

METHODS

Cellular Viability Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay was used to determine IC_{50} values for the platinum compounds. The HEI-OC1 cell line was grown in Dulbecco's Modified Eagle Medium (DMEM) with 50 units of γ-interferon per mL supplementation in 10 cm diameter plates in an incubator set to 33 °C and 10% CO_2. The cells were grown to 80-90% confluence, media was then removed from each dish, and 2 mL of 37 °C trypsin was added to each dish. The dishes were then placed in the incubator for 15 minutes. Next, 8 mL media was added to each dish, and cell clumps were broken up by up- and down-pipetting with a 5 mL pipette. Once the cells were thoroughly dispersed, the cell suspensions were transferred into a 50 mL conical centrifuge tube. A hemocytometer was then used to quantify cell density, and media containing 50,000 cells was added to individual wells in 24 well plates. These plates were then placed in the incubator for 24 hours. A concentration series (500, 50, 5, 0.5, 0.05 μM) of cisplatin, phenanthriplatin or pyriplatin (Figure 1) was then added to replicates of three wells for each concentration. Negative control wells (cells with media), positive control wells (cells with Triton X-100), and blanks (media only) were also prepared in triplicate. Plates were then placed in the incubator for 24 or 48 hours. Once incubation was complete, the MTT assay was performed for two hours, media was rapidly evacuated out of the wells, and MTT solubilization solution [10% Triton X-100 in acidic (0.1 N HCl) isopropanol] was added to each well for 15 minutes. A BioTek

Synergy HT (Winooski, VT) plate reader was then used to record formazan dye production at 570 nm and 690 nm absorbance wavelengths.

Reactive Oxygen Species Assay

ROS production in HEI-OC1 cells treated with pyriplatin, phenanthriplatin, and cisplatin was measured using flow-cytometry. HEI-OC1 cells were seeded in 6-well plates with 300,000 cells per well. Plates were then placed in the incubator for 24 hours. Wells were then treated in replicates of three with 24- or 48-hour IC_{50} values of the three platinum compounds, or with media only (negative control). Three wells were also prepared as no-dye controls (media only). After either 24- or 48-hours treatment time, media was aspirated from the wells and 2 mL of phosphate buffered saline (PBS) was used to wash the cells three times. 0.5 mL Accutase (Gibco, Gaithersburg, MD) was then added to each well to detach cells from the plates. Next, cells were added to micro-centrifuge tubes along with 1 mL of PBS each. Tubes were then centrifuged at 1000 rpm for 5 minutes before the supernatant was discarded and 500 µL of 10 µM ROS indicator dye (H_2DCFDA) (Invitrogen, Carlsbad, CA) in PBS was added to each tube, except that 500 µL of PBS with no ROS indicator dye was added to the no-dye control tubes. Next, the tubes were placed in an incubator set to 33 °C at 10% CO_2 for 30 minutes. They were then centrifuged again at 1000 rpm for 5 minutes. The supernatant was then removed, cells were washed with 1 mL PBS in triplicate, and 1 mL PBS was then added followed by resuspension of the cells. A Becton-Dickinson Accuri C6 (Franklin Lakes, NJ) flow cytometer was then used to analyze fluorescence emission, with the sample analysis limit

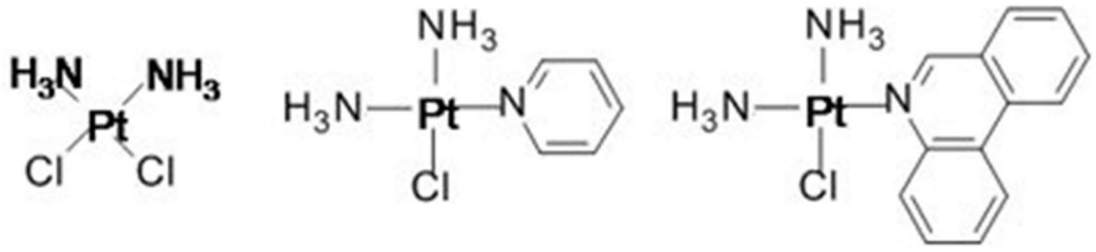
set to 5,000 events on the fast setting. Fluorescence values as a percent of the control were determined using data from the FL1 channel.

Caspase luminescence assays

Caspase-3/7 activity in treated HEI-OC1 cells was measured using a luminogenic kit (Promega, Fitchburg, WI). Media suspensions containing 10,000 cells were added to individual wells in white 96-well plates (Fisher Scientific, Waltham, MA). Plates were then incubated for 24 hours at 33 °C and 10% CO_2. Replicates of six were treated with either 24- or 48-hour IC_{50} value concentrations of the platinum compounds, media only (negative control), or 100 μM hydrogen peroxide (H_2O_2) (positive control). Six blank wells (media only) were also prepared. Plates were placed in the incubator for 24 or 48 hours and then luminogenic substrate was added to each well, and plates were left at room temperature in the dark for 0.5, 1, 2, and 3 hours before being read using the luminescent setting of a BioTek plate reader (Winooski, VT).

Statistical Analyses

Statistical analysis was performed using PRISM (GraphPad, version 6, La Jolla, CA). IC_{50} values for cisplatin, phenanthriplatin, and pyriplatin were calculated in PRISM using a sigmoidal, four parameter logistic equation. MTT assay standard deviation values were calculated using ED50 plus v1.0 online software (Mexico City, Mexico). For the ROS and caspase assays, a two-way ANOVA with Tukey's multiple comparisons test was performed; $p \leq 0.05$ was used as the level of significance.



cisplatin pyriplatin phenanthriplatin

Figure 1: The chemical structures of cisplatin, pyriplatin, and phenanthriplatin. Cisplatin is a bifunctional platinum-based chemotherapy drug that is effective against several forms of cancer but can also cause ototoxicity. Pyriplatin and phenanthriplatin are monofunctional platinum(II) complexes that have anti-cancer efficacy but whose ototoxicity is not well understood.

RESULTS

This project initially utilized the MTT spectrophotometric assay to measure the effect of cisplatin, pyriplatin, and phenanthriplatin on the cellular viability of HEI-OC1 cells. IC_{50} values (Table 1) show similar cellular viability for each compound tested at both the 24- and 48-hour timepoints. The IC_{50} values ± standard deviation for cisplatin were 1.66 ± 0.33 and 4.52 ± 0.17 for the 24 and 48-hour timepoints, respectively. The IC_{50} values ± standard deviation for phenanthriplatin were 1.64 ± 0.19 and 2.31 ± 0.29 for the 24 and 48-hour timepoints, respectively. The IC_{50} values ± standard deviation for pyriplatin were 11.11 ± 0.28 and 2.52 ± 0.11 for the 24 and 48-hour timepoints, respectively.

Next, flow cytometry using the fluorescent dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), was used to measure the relative ROS production of HEI-OC1 cells after treatment with IC_{50} values of cisplatin, pyriplatin, or phenanthriplatin. At the 24-hour timepoint, ROS production was 617.8% of the negative control for pyriplatin treatment, which is significantly elevated ($p < 0.001$) compared to the negative control. ROS production was 748.8% of the negative control for phenanthriplatin, which is significantly elevated ($p < 0.0001$) compared to the negative control. ROS production was 752.2% of the negative control for cisplatin, which is also significantly elevated ($p < 0.0001$) compared to the negative control (Figure 2). The positive control was 670.5% of the negative control. At the 48-hour timepoint, ROS production for each treatment

condition was not significantly different ($p > 0.05$) than the negative control (Figure 3). Pyriplatin was 118.0%, phenanthriplatin was 114.4%, and cisplatin was 111.8% of the negative control, respectively. The positive control was 225.0% of the negative control.

As a final step, a luminescent spectrophotometric assay was used to measure caspase-3/7 activity after cisplatin, pyriplatin, or phenanthriplatin treatment. At the 24-hour timepoint, caspase-3/7 activity was significantly lower for cisplatin ($p < 0.001$), pyriplatin ($p < 0.0001$), and phenanthriplatin ($p < 0.0001$) compared to the negative control (Figure 4). Percents of control were 85.7%, 81.7%, and 83.1%, respectively. At the 48-hour timepoint, the pyriplatin and phenanthriplatin treated samples had caspase-3/7 activity that was not different than the negative control. Percents of control for these treatment conditions were 101.4% and 89.0%, respectively. However, at 48 hours post-treatment, caspase-3/7 activity was significantly elevated ($p < 0.0001$) in the cisplatin treated samples compared to the negative control at this timepoint (Figure 5). The percent of control for cisplatin was 352.1%.

Table 1: MTT assay IC_{50} and standard deviation values for cisplatin, phenanthriplatin, and pyriplatin at 24 and 48 hours. The standard deviation value is provided after the ± symbol.

Treatment	IC_{50} (μM)
Cisplatin (24 Hr)	1.66 ± 0.33
Cisplatin (48 Hr)	4.52 ± 0.17
Phenanthriplatin (24 Hr)	1.64 ± 0.19
Phenanthriplatin (48 Hr)	2.31 ± 0.29
Pyriplatin (24 Hr)	11.11 ± 0.28
Pyriplatin (48 Hr)	2.52 ± 0.11

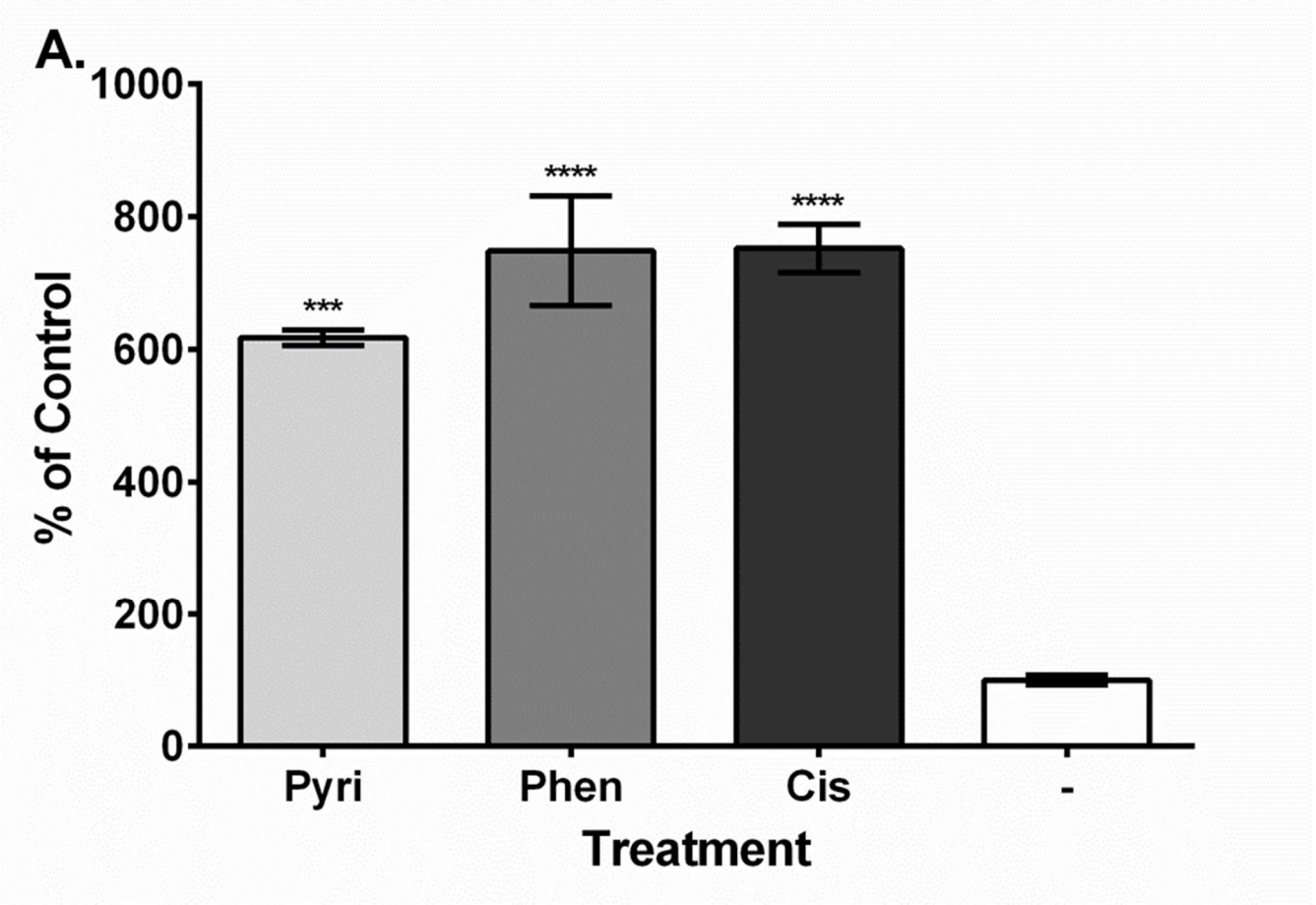


Figure 2: Cisplatin and the monofunctional compounds cause increased ROS production at 24 hours post-treatment. Abbreviation key: Negative control ("-"), pyriplatin ("Pyri"), phenanthriplatin ("Phen"), cisplatin ("Cis"). N=3. "***", $p < 0.001$; "****", $p < 0.0001$.

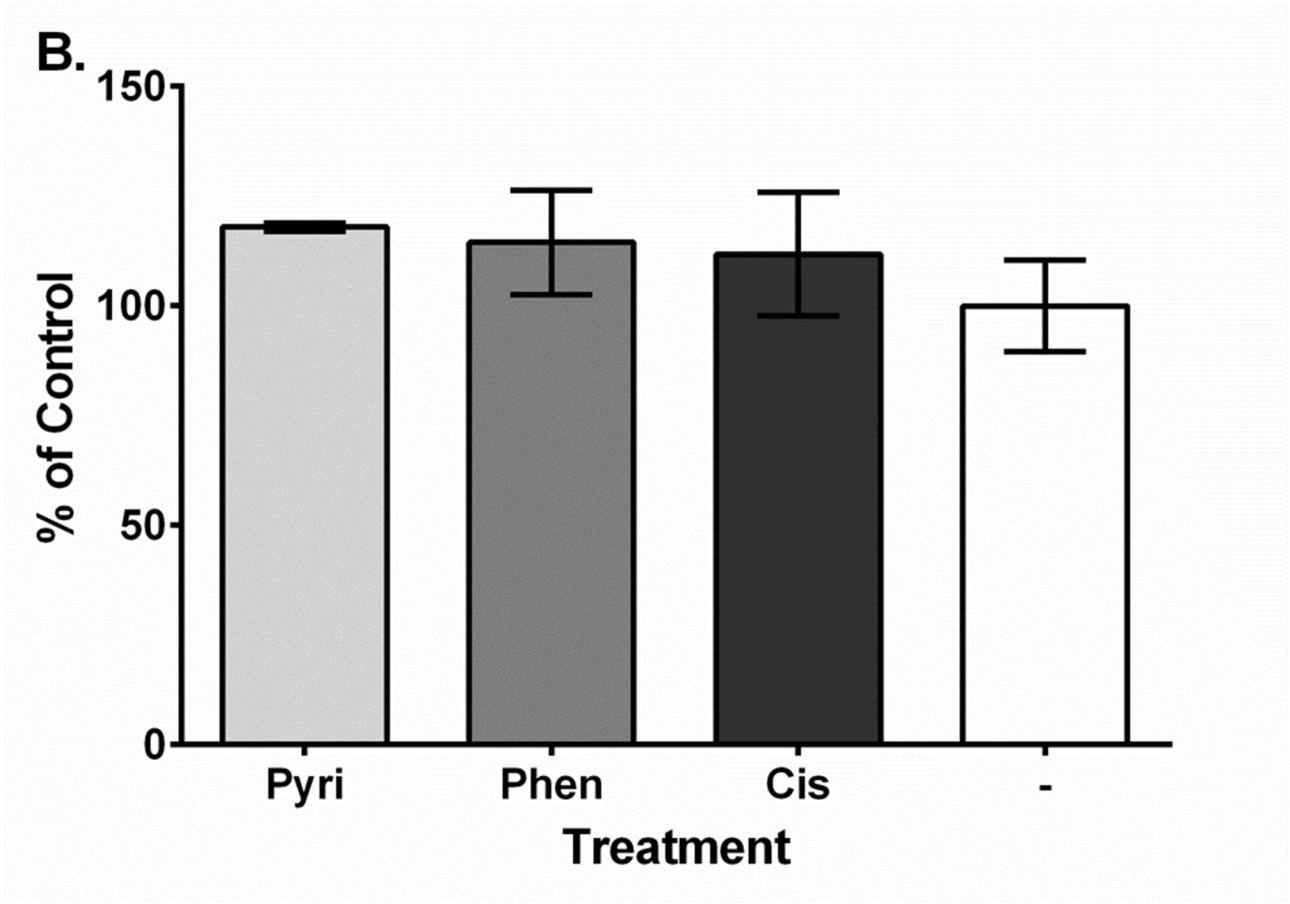


Figure 3: Cisplatin and the monofunctional compounds do not change ROS production at 48 hours post-treatment. Abbreviation key: Negative control ("-"), pyriplatin ("Pyri"), phenanthriplatin ("Phen"), cisplatin ("Cis"). N=3. $p > 0.05$.

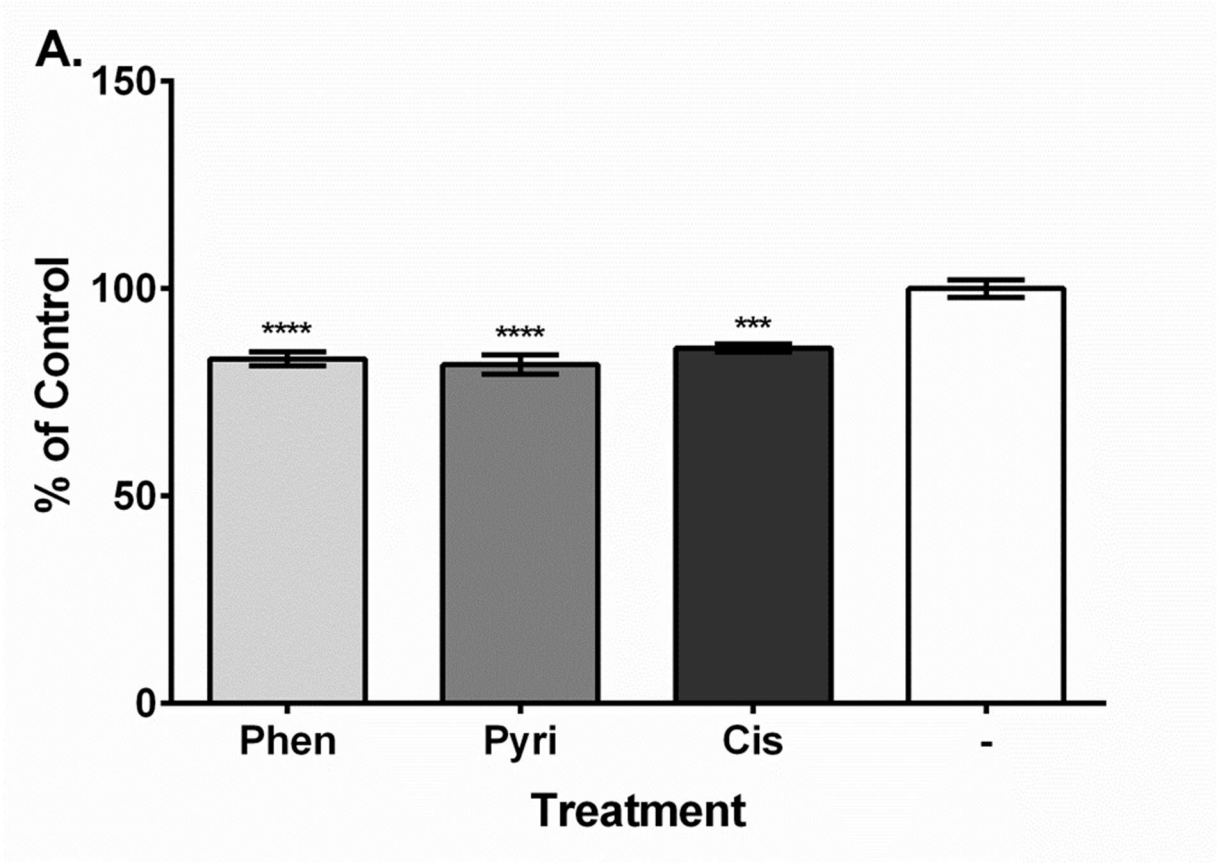


Figure 4: Cisplatin and the monofunctional compounds cause decreased caspase-3/7 activity 24 hours post-treatment. Abbreviation key: Negative control ("-"), pyriplatin ("Pyri"), phenanthriplatin ("Phen"), cisplatin ("Cis"). N=3. "***", $p < 0.001$; "****", $p < 0.0001$.

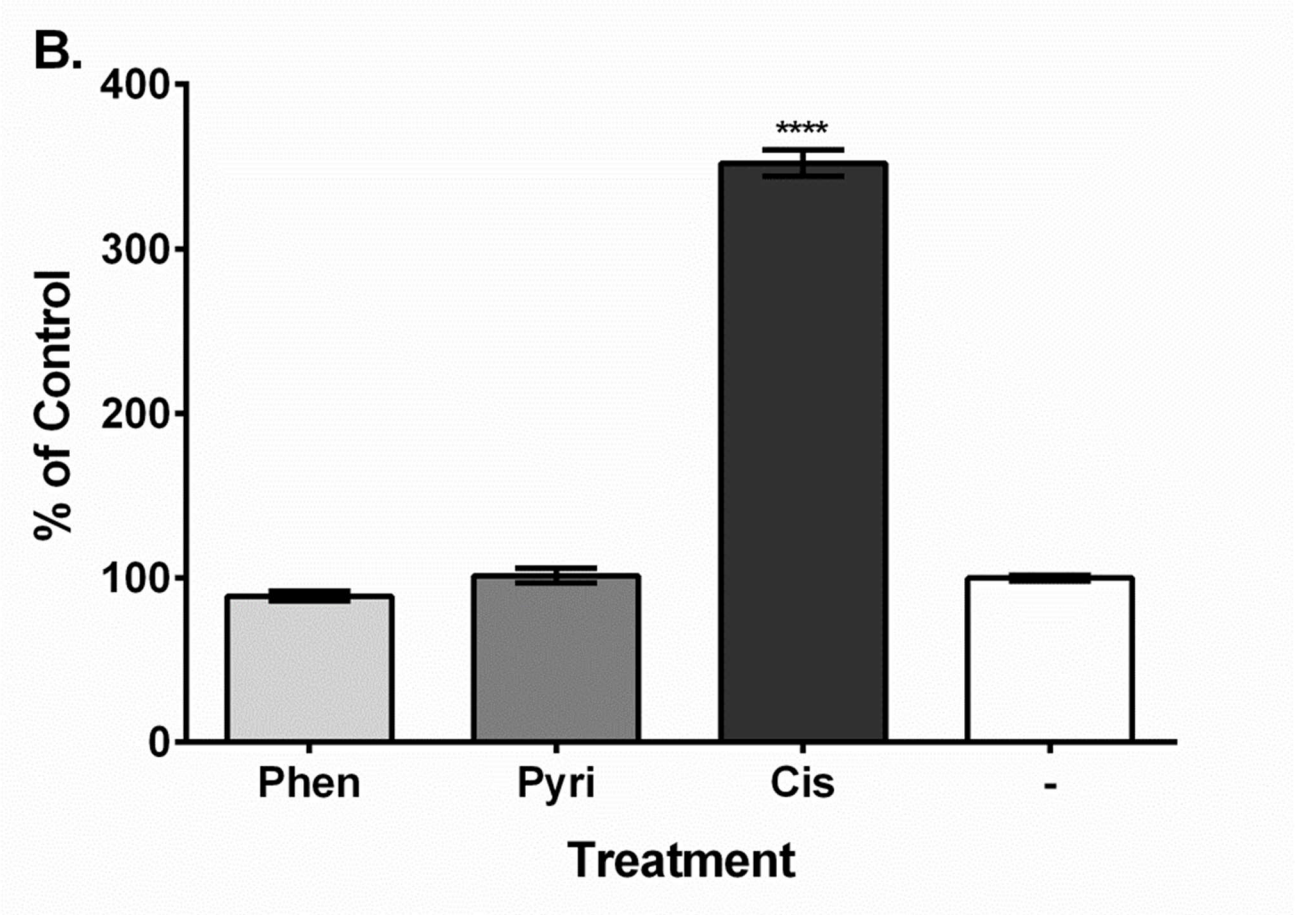


Figure 5: Cisplatin causes increased caspase-3/7 activity 48 hours post-treatment. Abbreviation key: Negative control ("-"), pyriplatin ("Pyri"), phenanthriplatin ("Phen"), cisplatin ("Cis"). N=3. "****", $p < 0.0001$.

DISCUSSION

Monofunctional platinum(II) compounds that signal through different cell death pathways than those used by cisplatin might have reduced side-effects in auditory cells. Cisplatin usually binds to DNA in the form of 1,2-intrastrand cross links between guanosine residues which causes DNA to bend followed by replication arrest, transcription inhibition, termination of the cell-cycle, and recruitment of proteins that initiate apoptotic signaling (Cepeda et al., 2007; Dasari and Tchounwou, 2014). Unlike cisplatin, phenanthriplatin and pyriplatin bind predominately to DNA guanosine residues without forming cross links or distorting DNA and act to inhibit transcription (Park et al., 2012; Riddell et al., 2016). As these monofunctional compounds may not activate some of the cell-death signaling pathways utilized by cisplatin, they may cause reduced ototoxicity compared to a bifunctional compound.

One mechanism that could be responsible for decreased cell viability is transcriptional blockage. Cisplatin adducts on DNA inhibit transcription in eukaryotic organisms and are correlated with anti-cancer efficacy (Todd & Lippard, 2009). Transcriptional inhibition can be from the blockage of transcription factors by platinum adducts, the formation of a physical barrier that prevents the movement of RNA polymerases, inhibition of chromatin remodeling, or some combination of the foregoing (Todd & Lippard, 2009). Arrested transcription can cause apoptosis by increasing p53 expression, as well as altering the balance between pro- and anti-apoptotic proteins

(Derheimer et al., 2005). Drugs utilizing a transcription inhibition mechanism may selectively target cancer cells due to constant activation of transcription factors, which promotes cancer cell death; whereas, transcription suppression has less of an effect in non-cancerous cells (Frank, 2009).

When cisplatin binds to DNA, about 90% of the lesions formed are 1,2-intrastrand crosslinks which cause the distortion of DNA (Huang et al., 1994). These lesions are most commonly repaired using the nucleotide excision repair (NER) pathway (Yimit et al., 2019). High mobility group-domain (HMG) proteins are typically recruited to 1,2-intrastrand crosslinks, and prevent DNA repair (Huang et al., 1994) possibly by hiding DNA adducts from repair enzyme recognition and stalling essential cellular operations (Cepeda et al., 2007). Like cisplatin, phenanthriplatin and pyriplatin can inhibit transcription (Kellinger et al., 2013), but DNA nucleotides bound with monofunctional platinum compounds should not bind to HMG proteins because of the lack of DNA distortion present (Johnstone et al., 2013).

When the MTT assay was used to measure the comparative effect on cell viability of cisplatin, phenanthriplatin and pyriplatin (Table 1), it showed that both monofunctional compounds suppressed viability to a similar degree as cisplatin. This result could mean that cisplatin and these monofunctional compounds can cause an equivalent level of transcriptional blockage, and hence cell-death, regardless of the presence of DNA distortion and the recruitment of apoptotic proteins to platinum-DNA adducts. However, the HEI-OC1 cell line used for this study is a hybridoma that combines a mouse cochlear auditory cell with a cancer cell and exhibits cancer cell-like proliferation (Kalinec et al., 2016). It is possible that cellular viability after treatment with

a monofunctional platinum compound would be much higher in normal auditory cells, which could exhibit lower levels of transcriptional blockage and cell death than in cancer cells or in modified cells that exhibit increased transcription.

Monofunctional compounds bound to DNA could also recruit different cell-death proteins than cisplatin, also causing decreased cell viability. Currently, research on monofunctional platinum(II) compound DNA adducts and their associated proteins is extremely limited. However, it has been shown that when cisplatin is bound to DNA, many proteins can be recruited to these adducts (Zeng et al., 2019). Therefore, it may be possible that when phenanthriplatin and pyriplatin bind to DNA nucleotides, unique proteins are recruited to these adducts which can signal into the same or different cell-death pathways that cisplatin utilizes. The similar IC_{50} values of the monofunctional compounds and cisplatin (Table 1) could suggest that novel DNA damage-associated proteins are involved in monofunctional platinum(II) compound cell-death signaling, similar to HMG proteins with cisplatin. Future studies would be needed to identify proteins which bind to monofunctional platinum(II) compound DNA adducts and to analyze their role in apoptosis.

However, the monofunctional compounds and cisplatin could have distinct transcriptional and downstream cell death mechanistic effects. Although cisplatin treatment can cause cell death as a result of interrupted transcription and DNA replication, it can also activate alternative or subsequent apoptotic pathways that cause even higher cytotoxicity, and which are responsible not only for increased anti-cancer efficacy, but also harmful side-effects (Marullo et al., 2013; Karasawa & Steyger, 2015).

Cisplatin cytotoxicity is typically associated with increased ROS generation that acts on multiple cellular targets (Marullo et al., 2011; Karasawa and Steyger, 2015; Gentilin et al., 2019). Cisplatin can upregulate some NADPH oxidase subunits and increase ROS production (Kim et al., 2010). Specifically, cisplatin treatment can increase activation of the NADPH oxidase isoform, NOX3, which then promotes signal transducer and activator of transcription-1 (STAT1) signaling, increased tumor necrosis factor-α (TNF-α) expression, and subsequent activation of the extrinsic apoptosis pathway (Kaur et al., 2011; Karasawa and Steyger, 2015). Cisplatin treatment can also prevent the action of cellular antioxidant systems and increase ROS production (Dammeyer et al., 2014). The monofunctional compounds and cisplatin caused increased ROS production at 24 hours (Figure 2), but ROS levels returned to normal at 48 hours (Figure 3). These results suggest that cisplatin and these monofunctional compounds may have a similar early temporal effect in HEI-OC1 cells to increase ROS production. This data set could also explain the elevated caspase-3/7 activity for cisplatin at 48 hours (Figure 5), as increased ROS at the earlier 24-hour time point could activate the extrinsic apoptotic pathway through STAT1 and TNF-α to subsequently increase caspase-3/7 activity. However, it is possible that the monofunctional compounds can activate caspase-3/7 signaling via the extrinsic pathway at a later time point.

As cisplatin-mediated DNA and ROS effects are early-stage components of cell death mechanisms, this project also investigated the effect of cisplatin, phenanthriplatin and pyriplatin on the activity of caspases-3/7, which are late-stage effectors of apoptosis in auditory cells integrating DNA damage, intrinsic and/or extrinsic cell-death pathways (Devarajan et al., 2002; Karasawa and Steyger, 2015; Sheth et al., 2017). Interestingly, at

24 hours, cisplatin and both monofunctional compounds caused decreased caspase-3/7 activity (Figure 4), which could be a compensatory effect in response to the elevated ROS levels. At the 48-hour time point, cisplatin-treated samples had increased caspase-3/7 activity, while the monofunctional compounds were not different from the control (Figure 5). This data set suggests that cisplatin might cause apoptosis after an early phase of ROS production (Figure 2) followed by subsequent increased caspase-3/7 activity (Figure 5), perhaps through one or more cell-death pathways, or that the monofunctional compounds activate caspases-3/7 at a later time point.

It is possible that cisplatin and the monofunctional compounds could act through pathways other than the ones studied in this project. Cisplatin treatment of HEI-OC1 cells can cause cell death by promoting the release of apoptosis inducing factor (AIF) (Lee et al., 2011). AIF can be released from the mitochondria when ROS levels are high (Redza-Dutordoir & Averill-Bates, 2016). Additionally, cisplatin-mediated DNA damage in models of nephrotoxicity can cause activation of p53-induced protein with a death domain (PIDD), which can cause mitochondrial release of AIF and cell death independent of caspase-3/7 activation (Karasawa and Steyger, 2015). As our earlier 24 hour time point data (Figure 2) indicates increased ROS levels from monofunctional platinum(II) compound treatment, it is possible that phenanthriplatin and pyriplatin could signal through AIF instead of caspase-3/7, which could instead be associated with cisplatin cell death signaling (Figure 5). Cisplatin treatment can also cause endoplasmic reticulum stress in auditory cells and increased caspase-12 expression as part of a cell death mechanism independent of the extrinsic and intrinsic apoptosis pathways (Nakagawa et al., 2000; Karasawa and Steyger, 2015; Zong et al., 2017). As caspase-12

signals through caspase-3 (Rao et al., 2006), our data might support a role for caspase-12 with cisplatin as caspase-3/7 activity only increased at 48 hours after treatment with this compound (Figure 5). However, it is possible that the monofunctional compounds could signal through caspase-12 at a later time point than those measured in this project.

Studies of candidate otoprotectants to prevent the ototoxic side-effects of cisplatin treatment have identified many compounds, including antioxidants and inhibitors of caspase-3 (Paksoy et al., 2011; Sheth et al., 2017). The results from my ROS experiments (Figures 2 and 3) suggest that increased levels of ROS may be responsible for the reduced cellular viability observed with the monofunctional compounds (Table 1). Therefore, otoprotectants aimed at reducing ROS could have beneficial effects for monofunctional as well as bifunctional platinum(II) compounds. However, this study cannot rule out a role for caspase inhibitors or drugs directed at other molecular targets as potential otoprotective agents for use with phenanthriplatin and pyriplatin.

In conclusion, the monofunctional platinum(II) compounds, phenanthriplatin and pyriplatin, may cause ototoxicity to a similar extent as cisplatin. Both monofunctional compounds may promote ototoxicity by initially elevating ROS production, but only cisplatin may cause cell death through caspase-3/7 signaling. My results indicate that these compounds may utilize different cell death mechanisms, but additional research would be required to clarify the mechanistic action of these monofunctional compounds. Future studies could be conducted in HEI-OC1 cells at other time points or could explore other cell death signaling mechanisms, e.g., those incorporating AIF or caspase-12, to provide additional data to elucidate whether these monofunctional platinum compounds utilize different cell-death signaling than cisplatin. Once a more complete understanding

of monofunctional platinum(II) compound cell-death signaling is obtained, it may be possible to develop chemotherapeutic agents that counteract this signaling in auditory cells while simultaneously retaining the anti-cancer efficacy of these platinum compounds.

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